DETECTION OF TRICLOSAN IN CENTRAL VIRGINIA WASTEWATER AND IDENTIFICATION OF RESISTANT MICROORGANISMS

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By

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ABSTRACT

Triclosan (5-chloro-2(2,4-dichlorophenoxy)phenol) or (Irgasan® DP 300) is an antimicrobial agent typically used in personal care products to prevent microbial growth and ultimately gets dispersed into the environment. Pharmaceuticals and personal-care products (surfactants, antibiotics, and antimicrobial agents), collectively known as PPCPs, are considered emerging contaminants due to their potential toxicological effects in the environment. This study determined the presence of triclosan in primary influent wastewater at two Central Virginia wastewater treatment plants. Concentrations of triclosan varied from 0.6 to 116 ug/L using HPLC analysis. The identification of triclosan-resistant bacteria in the influent were found using BIOLOG biochemical substrate testing. Several identified strains included *Serratia rubidaea, Corynebacterium nitrilophilus, Brevibacterium mcbrellneri, and Burkholderia glumae.*

INTRODUCTION

Xenobiotics in the Aquatic Environment

The detection of xenobiotics in the environment has been given much attention recently.[1, 2] Examples of xenobiotics include pesticides, fertilizers, detergents, plasticizers and pharmaceuticals.[3] Xenobiotics, synthetic organic compounds, have been detected in surface waters, ground waters, and sewage treatment plant effluents.[1, 3, 4] Detection of pharmaceuticals in the aquatic environment are of particular concern due to the potential health risks associated with them. Designed for a specific effect, pharmaceuticals are made to evoke a desired response through anabolism, chemical reactions, or other metabolic pathways. They are engineered to penetrate cellular membranes, to be active at micromolar or nanomolar

concentrations, and to remain stable at varying pHs. It is this bioactivity and specificity that makes them efficacious and potentially dangerous to organisms.

Pharmaceuticals enter the environment through excretion following therapeutic use, discharge of treated wastewater, and by disposal of unused medications by consumers.[2] Many xenobiotics are released into the environment due to the inability of the wastewater treatment plants (WWTPs) to remove them.[1,5] Pharmaceuticals and other personal-care products (surfactants, antibiotics, and antimicrobial agents), collectively known as PPCPs, are not mandated under the U.S. Clean Water Act for removal from wastewater.[5] Triclosan was found in wastewater (0.07-14000 μg/L), in streams (50-2300 ng/L), in seawater (50-150 ng/L) and in sediments (1-35 μg/kg).[6] The annual volume of raw sewage entering surface waters is approximately 860 billion gallons.[7] Approximately seven percent of the total volume of U.S. wastewater enters the environment untreated.[7]

Triclosan: Physical Properties

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol or Irgasan® DP 300) is an antimicrobial agent typically used in personal care products such as toothpastes, soaps, shampoos, and deodorants.[8-11] A chlorinated biphenyl ether, triclosan is lipophilic and relatively stable, despite the phenolic hydroxyl group (Figure 1).

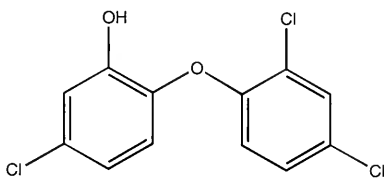


Figure 1. Structure of Triclosan [11]

Table 1. Key summary of the physicochemical properties of triclosan.[12]

Property	Value
Molecular weight	289.6
Water solubility	12 mg/L
Dissociation constant (pKa)	8.14 @ 20 °C
Vapor Pressure	7x10-4 Pa at 25 °C
Partition coefficient (log K_{ow})	4.8
Aerobic biodegradation in soil	17.4 to 35.2 day half-life
Aqueous photolysis	41-min half-life at pH of 7.0 and 25 °C
Adsorption to suspended solids (Koc)	47,454 ml/g

Fate of Triclosan in the Aquatic Environment

There is reason for concern that triclosan and other PPCPs may have significant impact on the environment.[12] The degradation characteristics of many of these compounds are unknown along with the chemical properties of their degradants. While studies on the degradation characteristics of triclosan have been conducted, very little is known about how these breakdown products and conjugates affect the receiving ecosystems.

Triclosan is an antimicrobial agent designed to kill a wide range of "undesirable" microorganisms. Its introduction into the water may result in the unintended targeting of other, beneficial microorganisms.[13] Several strains of microorganisms may gain resistance due to the long term exposure of triclosan at increasingly high concentrations.[14] It is often

assumed that hospitals are the main source for input of resistant bacteria into the influent, yet because of the use of antibiotics at home it is more probable that the general community is responsible for the main input of resistant strains.[15]

Triclosan was initially thought to attack the plasma membrane making it more porous and preventing nutrient uptake and growth.[16] That mechanism of action justified the increased use of triclosan as an antimicrobial agent in personal care products since its target in organisms was a non specific disruption in the membranes.[16] McMurray *et al.* were the first to discover the specific mechanism behind triclosan's ability to kill bacteria through inhibition of the *fabI* gene rather than just being a general "biocide."[17] Triclosan has been found to inhibit the enoyl-acyl carrier protein reductase (FabI), which is involved in fatty acid synthesis.[17] The last enzyme required for the synthesis of lipids is typically FabI, encoded by the *fabI* gene. Crystallographic data proves that triclosan binds to FabI when NAD+ cofactor is present.[18] X-ray crystallographic data were obtained showing that the triclosan bound through H-bonding and hydrophobic forces to the FabI-NAD+ complex and formed a stable ternary complex.[19] The enol rings of triclosan form a π-π stacking with the nicotinamide ring, while the hydroxyl group on the phenolic ring hydrogen bonds to the hydroxyl group of nicotinamide ribose and to the phenolic oxygen of the tyrosine 156 residue.[20] The occurrence of the stacking interaction in the ternary complex contributes to the enhancement that triclosan and NAD+ show in their binding affinities.[21] Triclosan interacts with the NAD+, essentially binding to the enzyme-substrate complex, causing inhibition. Research has also demonstrated that mutations in the active site of *fabIp* disrupted the formation of the ternary complex and resulted in acquired resistance to the antimicrobial agent.[19]

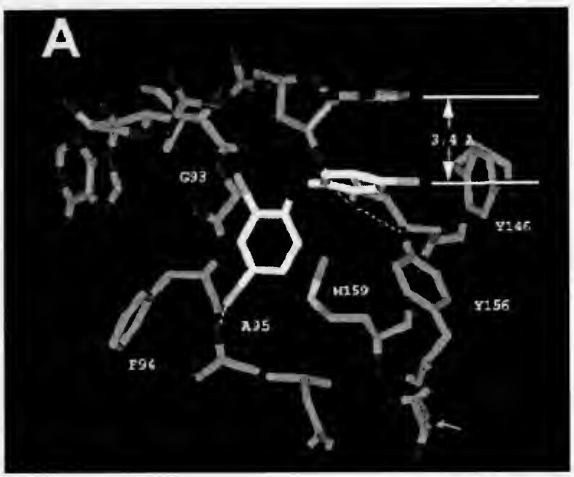


Figure 2. Structure of the FabI (green)-NAD+ (organge) and triclosan (white) ternary complex and active site.[19]

The detection of triclosan in the aquatic environment has been shown to disrupt algal community growth and structure.[22] An increase in the concentration present resulted in a biomass reduction and genus diversity reduction for the algae.[16] This reduction could ultimately have an impact on the nutrient levels and processing capacity as well as the food web.

Laboratory experiments have shown that the dissociated phenoxide form of triclosan rapidly decomposes in surface waters having a pH 8.0 when exposed to sunlight.[23] However, the nonodissociated form and methylated triclosan remained stable during photodegradation.[23] The formation of methylated triclosan is thought to be formed during the biological methylation in plants. The fate of these two compounds is very different. Triclosan's susceptibility to degradation by photolysis suggests a smaller bioaccumulation whereas the

persistence of methyl triclosan leads to greater potential in bioaccumulation in fish and other aquatic species.[24]

Triclosan is both acutely and chronically toxic to aquatic systems, though it appears nontoxic to mammals. The effective concentration of triclosan at which 50% of the population is effected (EC_{50}) and no-observed-effect concentration on the population (NOEC) have been reported for fathead minnow (*Pimephales promelas*), water flea (*Daphnia magna)*, bluegill sunfish (*Lepomis macrochirus*), and rainbow trout (*Oncorhynchus mykiss*).[12, 22] The activity level, startle response, growth rate and survival of *Rana pipiens* tadpoles has also been reported to decrease due to the impact of triclosan.[25]

Although direct photolysis has been suggested as the main degradation pathway, adsorption of triclosan into activated sludge has also been designated as a main pathway due to triclosan's hydrophobic nature.[26] Other laboratory experiments have shown the rapid oxidation of triclosan by manganese oxides (MnO_2 and MnOOH).[26] Metals such as Mn, Fe and Al are commonly be found in the soil. The results of those experiments support the mechanism that triclosan is oxidized to a phenoxy radical followed by radical coupling and then finally further oxidation to form the products shown in Figure 3.

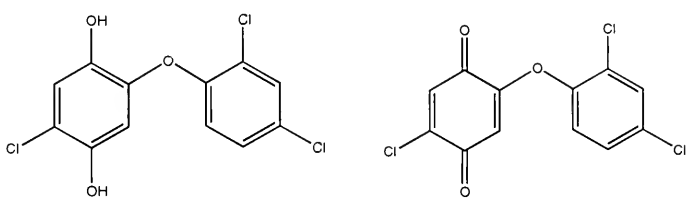


Figure 3. Formation of oxidized metabolites of triclosan.[26]

A study has also been conducted to characterize the kinetics and products of triclosan and free chlorine under the conditions of drinking water treatment. Triclosan rapidly degraded in the presence of free chlorines.[27] Results of the study indicated the formation of chloroform, 2,4-dichlorophenol, 2,4,6-trichlorophenol, and several other chlorinated triclosan intermediates.[28]

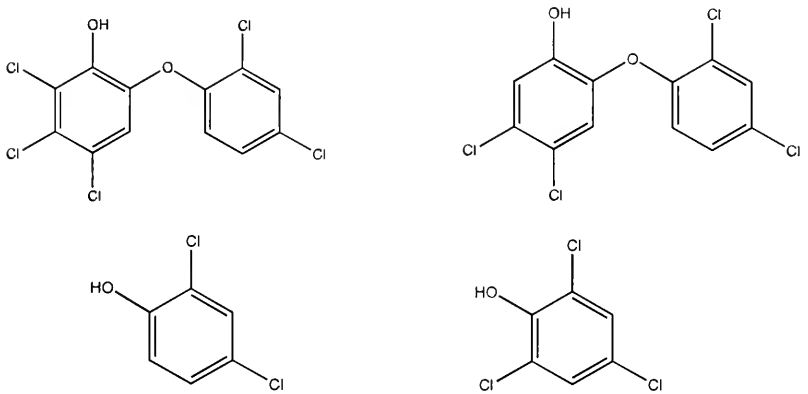


Figure 4. Formation of chlorinated metabolites of triclosan.[27,28]

With the use of triclosan for more than thirty years, much research has accumulated on the compound including Nuclear Magnetic Resonance (NMR) spectroscopy. Data has been compiled for methyl triclosan[29], triclosan embedded within egg yolk lecithin model membranes[30], the triclosan metabolite 2,8-dichlorodibenzo-p-dioxin[31], formed aggregates of triclosan with cyclodextrins[32], but has done so primarily through the chemical shift data. A complete proton and carbon assignment for just triclosan has yet to be reported.

Rationale for Research

With such a concern for the effect of triclosan in the aquatic environment, two Central Virginia wastewater treatment plant sites were analyzed for their concentration of triclosan present in the samples and the variability in their concentrations were compared. The isolation and identification of triclosan-resistant microorganisms from the wastewater samples were also assessed.

MATERIALS AND METHODS

Wastewater Collection

Four, 1-liter samples of influent wastewater were collected from the wastewater treatment plant of Amherst, Virginia, and the Rivanna Wastewater Treatment Plant at Moores Creek in Charlottesville, Virginia. These two WWTPs were chosen as sampling sites due to the significant differences between them while remaining in close proximity to each other. The Amherst WWTP is flow volume dependent because of the relatively small population that utilizes the facility. The average flow volume of Amherst is on the order of one million gallons per day. Conversely, the Charlottesville WWTP's volume flow remains relatively constant throughout the course of the year due to the relatively large population of Albemarle County and also from the contribution of the University of Virginia and its Medical Center. Once collected, the samples were transported back to laboratory in a cooler with ice and stored at 4 °C for no longer than twenty-four hours prior to extraction.

Sample Extraction

The samples were first centrifuged at 2000 *g* for 25 minutes in to remove particulate matter. The supernatant was passed through a Waters Oasis HLB C_{18} 3 cm^3/60 mg sorbent

solid-phase extraction (SPE) cartridge (Waters Corporation, Milford, Massachusetts). The cartridge was previously conditioned with 1 mL of methanol and equilibrated with 1 mL of ultrapure water. Once half of the entire sample, 0.5 liter, had passed through the SPE using a vacuum manifold pump, it was eluted with 4 mL of 50:50 methanol:acetone containing 10 mM acetic acid. The eluents were allowed to evaporate under a hood overnight and then reconstituted in 1 mL of the aforementioned solvent. They were then filtered using a Gelman Acrodisc 13CR PTFE 0.2 µm filter with a sterile B-D 1cc 26G 3/8 syringe and Precision Glide Needle into Target DP C4000-1W HPLC vials.

Sample Analysis by HPLC

An Agilent 1100 Series HPLC was used to determine the presence of triclosan in the wastewater treatment plant samples. A Phenomenex 5 µm, 150 x 4.60 mm column was used to separate triclosan from the other extracted compounds. The samples were eluted off the column using an isocratic method with a mobile phase of HPLC grade acetonitrile (Merck; Darmstad, Germany) and 0.1% (v/v) acetic acid in the HPLC grade water (Mallinckrodt Inc, New Jersey). The mobile phase was held at 60:40 acetonitrile:water for 30 minutes. After the sample had eluted off the column, the mobile phase was changed to 100% acetonitrile in order to flush the column of any remaining compounds prior to the analysis of the next sample. A flow rate of 1.0 mL/min with an injection volume of 100 µL was used. Elution was monitored using the diode array detector at 230 nm. Previous research has determined the absorption wavelength of triclosan to range from 230-282 nm.[14, 33] The photodiode array can be used to identify triclosan through comparison of the absorption spectra. The samples were analyzed in triplicate and a mean solution concentration and a standard deviation were calculated. The

sample chromatograms of the triclosan peaks were compared to the reference standard (Ciba Specialty Chemicals, High Point, NC).

Quantification of Triclosan in Samples

Standards of varying concentrations 0.1, 0.3, 0.5 and 10 ppm were analyzed using HPLC. Area counts for each standard were plotted against the respective concentration. This information was then used to generate a standard curve and equation. The area counts for the peaks from the samples were determined using the Agilent ChemStation Software. Triclosan concentrations in the wastewater samples were derived using the linear equation from the standard curve.

Isolation of Triclosan-Resistant Microorganisms

One liter influent wastewater samples were collected from both the Amherst County Wastewater Treatment Plant and the Rivanna Wastewater Treatment Plant at Moores Creek in Charlottesville, Virginia. The samples were aerated and given a tryptone/peptone nutrient broth (Difco) prior to plating. Each wastewater sample was diluted in phosphate buffered saline (PBS) buffer (1:100) and a 150 μL aliquot was spread onto R2A (Difco) agar plates. The plates contained concentrations of 10, 15, 20, 50 and 100 mg/L of triclosan using acetone as the carrier solvent. The control plates contained an equivalent volume of acetone as the triclosan test plates. The plates were incubated at 35 °C for 24 hours. Isolated colonies growing on these plates were then identified.

Identification of Triclosan-Resistant Organisms

Triclosan resistant strains were identified using the BIOLOG identification system and procedures (BIOLOG, Inc., Hayward, California). Individual strains were stained in order to determine whether they were Gram-positive or Gram-negative and their morphological characteristics. They were also subjected to an oxidase test and a Triple Sugar Iron (TSI) test as further characterization. The oxidase BBL *Dryslides*® tested for the utilization of cytochrome while the TSI slants recognized the fermentation of glucose, sucrose and lactose and if catabolism was performed aerobically or anaerobically. Inoculums of the strains were prepared according to the specified density ranges and plated onto the GN2 or GP2 MicroPlates®, depending on the results of the Gram-positive/negative test. The MicroPlates are a 95 carbon source utilization test plus control. The results of the staining, oxidase, and TSI tests, along with that of the MicroPlates® were entered into to the BIOLOG databases and the identities of the strains were determined through multivariate statistical analysis.

NMR Analysis of Triclosan

One- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy of triclosan was obtained using a JEOL ECX 400 MHz spectrometer. The triclosan standard was dissolved in $CDCl_3$ (Cambridge Isotope Laboratories). The proton spectra (400 MHz) was referenced to residual $CHCl_3$ at 7.23 ppm while carbon (100 MHz) was referenced to $CDCl_3$ at 77.33 ppm. Cadmium nitrate (Flinn Scientific) was subsequently added to determine the corresponding assignment of the OH signal.

RESULTS

Characterization of Wastewater

The microflora present in the wastewater were visually identified underneath the light microscope (Figure 5).[34] A Standard Reference Material (SRM) was used to generate a standard curve for the elements: zinc, copper and nickel by atomic absorption spectroscopy (AAS) (Figure 6). The R^2 value for the standard curve was 0.982. Each wastewater sample was analyzed by the AAS. The concentrations of both copper and nickel were below the detection limits of the AAS. Using the standard curve, the average concentration of zinc was quantified using the standard curve (Figure 7).

Quantification of Triclosan Concentrations

The triclosan concentrations from the influent wastewater were measured through HPLC analysis. The standard triclosan concentration was injected into the HPLC and utilized the mobile phase of 60:40 acetonitrile:water.[14] The standard had an average retention time of 10.4 minutes (Figure 8). A standard curve was generated using the area counts of the chromatograms of the known triclosan concentrations (Figure 9). The R^2 of the standard curve was 0.997. Representative chromatograms for each sample site were recorded (Figure 10 & 11). Representative chromatograms for each sample site with a spike were recorded (Figure 12 & 13). The concentrations of the triclosan were then calculated using the standard curve (Table 2).

Isolation and Identification of Triclosan-Resistant Microorganisms in Wastewater

The control plates showed the most bacterial colony growth. A small number of isolated colonies were visible on the Amherst 10 and 15 ppm triclosan enriched plates (Figure 14). A larger number of isolated colonies were also visible on the Charlottesville 10, 15, 20, and 50 ppm triclosan enriched plates (Figure 15).

Triclosan resistant isolated colonies were subjected to Gram-staining, an oxidase test, and a triple sugar iron (TSI) test (Table 3). The strains were identified using BIOLOG protocol (Table 4).

NMR Assignments

Complete proton and carbon assignments were made for triclosan (Table 5). The molecular modeling program Spartan '04 was also used to generate a theoretical NMR spectra and assignment table (Table 6). A comparison of the two methods' assignments, actual by the JEOL ECX 400 MHz and theoretical by Spartan '04, was also performed (Table 7). The ^{1}H NMR spectrum of triclosan was recorded (Figure 16). The ^{13}C NMR spectrum of triclosan was also performed (Figure 17). A DEPT 135 was run on triclosan (Figure 18) along with a ^{1}H-^{1}H COSY (Figure 19). Next, a nOe irradiation was recorded (Figure 20). It was then followed by an HMBC (Figure 21) and then another HMBC with the addition of $Cd(NO_3)_2$ (Figure 22).

DISCUSSION

Characterization of Wastewater

The diversity of the microflora present in the wastewater samples from Amherst were of interesting note. The identified species were varied and included: *Daphnia* (Phylum- Arthropoda), *Microthamnion* (Phylum- Chlorophyta), and *Epiphanes* (Phylum- Rotifera). Such diversity in the aquatic system usually indicates that the overall environment is healthy. [35,36] Significant concentrations of zinc were found in the wastewater samples from both Amherst and Charlottesville. The average calculated concentration of zinc for Amherst was 908.8 ppb while for Charlottesville the average zinc concentration was found to be 899.7 ppb. The higher concentration found in the Amherst wastewater plant was initially surprising due to its smaller size, but once the Buffalo Air Handling Company was identified as using large quantities of zinc in their industrial process was accounted for, which also service this treatment plant, the data seem logical. Both the concentrations of nickel and copper for both treatment plants were below the detection limits of the atomic absorption spectrometer.

Quantification of Triclosan Concentration Determined in Wastewater

The detection of triclosan in the influent wastewater samples demonstrates the use and subsequent release into the environment of triclosan as an antimicrobial agent in personal care products. The greatest concentration of triclosan was found in the Moores Creek Rivanna WWTP in Charlottesville, Virginia. The mean concentration overall from both sampling months was 39.1 ug/L +/- 48.9. While less triclosan was found at the Amherst facility, it does not depreciate the significance of its presence there. In analyzing the individual mean concentrations from the WWTPs for each month, specifically at the Moores Creek Rivanna

WWTP in Charlottesville, Virginia there much fluctuation from month to month which could take into account the overall standard deviation being larger than the overall mean.

The concentrations of triclosan varied at the two wastewater treatment plants over the two month collection time period. Such variation provokes speculation into the uses of triclosan. As initially hypothesized, the Moores Creek Rivanna Wastewater treatment plant had the greater concentration of triclosan due to it receiving contributions from both the University of Virginia and the surrounding medical facilities.

The overall fate of triclosan in the environment is just slowly being elucidated. The toxicity level of triclosan against certain aquatic organisms has been tested.[22] *Anabaena flosaquae*, algae, has demonstrated a 96-hour EC_{50} of 1.6 ug/L; larger concentrations of triclosan showed almost no growth.[22] While the mean Amherst concentration is below this value, the Moores Creek Rivanna Wastewater treatment facility is almost twenty-five times the tested EC_{50} value. Such a high concentration at the Moores Creek Rivanna WWTP could lead to a reduced amount of algae present in the wastewater.

With triclosan having a pKa value of 8.1, it readily ionizes at most pHs found in the environmental water supplies. The neutrality of triclosan allows it to permeate easily across a plasma membrane where an ionized compound would simply be stopped. The passage of triclosan into the cytosol may prove to be toxic to more aquatic organisms at higher concentrations.

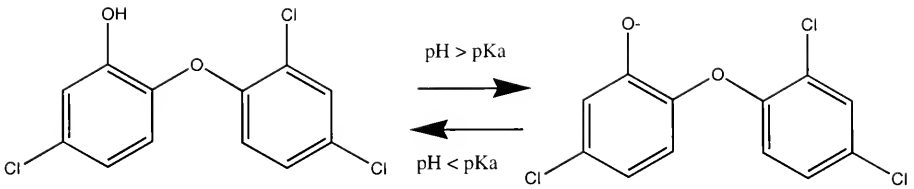


Figure 23. Ionized and neutral form of triclosan.[23]

No data exist to date that demonstrates triclosan is toxic to humans, but trace amounts have been found in human blood samples[37] and in mothers' breast milk.[38]

Triclosan, as an antimicrobial agent, and other PPCPs are just now becoming recognized by the EPA as a potential health and environmental concern. Research into the mechanism of triclosan's inhibition first showed deactivation of a protein associated with fatty acid biosynthesis.[17] This means of binding is currently under investigation as recent works have shown that a direct relationship exists between *fabI, vac7* and *vac14*.[39-42] Both the *vac7* gene product (vac7p) and *vac14* gene product (vac14p) serve as regulators of the *fabI* gene product, fabIp. A specific mechanism of inhibition threatens the potency of triclosan as the potential exists for more bacteria to gain resistance to it, other antimicrobial agents, and even antibiotics. If the concentrations of triclosan and other PPCPs in wastewater continue to increase without an established baseline of the present conditions in place, it is conceivable that such an increase could contribute to the increase in bacterial resistance before the public is made aware of the issue.[43]

Isolation and Identification of Triclosan-Resistant Microorganisms in Wastewater

Several isolated strains were identified on triclosan-enriched agar plates. Many of these strains have shown resistance to antibiotics and capabilities of degrading very harmful chemicals including PCBs.[35,36] The isolated triclosan-resistant bacteria included: *Serratia rubidaea, Corynebacterium nitrilophilus, Brevibacterium mcbrellneri, Brevundimonas vesicularis, Raoultella (Klebsiella) planticola, and Burkholderia (Pseudomonas) glumae.*

Serratia rubidaea was isolated from a Charlottesville wastewater sample. It is a Gram-negative bacteria commonly found in human blood and bile.[35] The genus is one of the free-living members of the family Enterobacteriaceae.[36] Individuals having compromised immune systems do pose a risk of having a very serious infection from this opportunistic pathogen if exposed to it.

Corynebacterium nitrilophilus is a Gram-positive bacteria also isolated from the Charlottesville wastewater. The species is non-motile, lack capsules and do not form spores.[36] They are known to degrade acetonitrile and other organic solvents.[35]

Brevibacterium mcbrellneri like *Corynebacterium nitrilophilus,* is a Gram-positive bacteria isolated from Charlottesville wastewater. The species can be infectious towards humans with compromised immune systems.[35]

Brevundimonas vesicularis was isolated from the Charlottesville wastewater sample as well. It is a Gram-negative bacteria that can be found in aquatic systems. It is also known to be resistant to antibiotics like ciprofloxacin. [35]

Raoultella (Klebsiella) planticola was isolated from a Charlottesville wastewater sample. It is a Gram-negative bacteria having flagella and a very large capsule that is known to degrade antimicrobial agents, including triclosan.[36]

Burkholderia (Pseudomonas) glumae is a Gram-negative, aerobic, motile bacteria which was isolated from an Amherst wastewater sample. The species is commonly found in aquatic systems and throughout nature and can degrade poly chlorinated biphenyls (PCBs). [36]

The variety of bacterial strains identified demonstrating resistance to triclosan is a concern. These microorganisms possess many capabilities in resisting the inhibitory effects of triclosan which happen to include: genetic mutations, enzymatic degradation, and chemical modification. Whatever mechanism is utilized, gained bacterial resistance is a potential threat to public health.[43]

NMR Assignments

First glance of the ^{1}H NMR spectrum (Figure 16) revealed seven signals (minus the $CDCl_3$ peak at 7.23 ppm). These seven peaks integrated to the seven protons of triclosan. A quick glance was then made at the ^{13}C NMR spectrum (Figure 17) (minus the $CDCl_3$ triplet at 77.33 ppm). It confirmed twelve carbon signals and supported the molecular formula of

triclosan: $C_{12}H_7O_2Cl_3$. The DEPT 135 (Figure 18) proved the absence of methylenes, and the presence of six methine carbons and six quaternary carbons in the aryl range. The coupling constants and multiplicities from the integral expansions were determined for all of the proton peaks and were consistent with what was expected.

Attention was then turned toward the ^1H-^1H COSY NMR spectrum (Figure 19). The presence of the bridging oxygen between the two aromatic rings should seperate the ring-ring system communication. The three signals: 6.65, 6.8 and 7.05 ppm, integrated to three protons and communicated to no others. Being positioned relatively upfield, they were assigned to the A ring of triclosan since the contribution of electron density into the ring system by the OH group which tends to push the chemical shift upfield.[44] The signal at 7.05 ppm was tentatively assigned H-1 due to its splitting pattern as a doublet and its long range meta coupling of 2.29 Hz to the signal peak at 6.8 ppm. Meta coupling typically ranges from 1-3 Hz.[44] From H-1, the signal at 6.8 ppm was assigned to H-5 due to its splitting pattern being a doublet of doublets and both its close 2.29 Hz (orthro, J=6-10 Hz) and long 8.24 Hz (meta, J=1-3 Hz) range coupling. The COSY experiment then confirmed the communication of 6.65 ppm, designated H-4, to H-5 thus completing the proton assignment for the A ring.

The three aromatic proton signals: 6.9, 7.2 and 7.4 ppm, integrated to three protons and comprised the second aryl spin system. They were positioned relatively downfield due to the deshielding effects of the disubstituted chlorines on ring B.[44] The signal at 7.49 ppm was assigned H-9 due to its splitting pattern as a doublet and its long range meta coupling of 2.29 Hz to the signal at 7.2 ppm. Working from H-9, the signal peak at 7.2 ppm was designated as

H-11 due to its splitting pattern being a doublet of doublets and both its short and long range J coupling. The peak at 6.9 ppm was then assigned as H-12 because of its communication to H-11 on the COSY, thus completing the proton assignments for ring B.

The nOe irradiation (Figure 20) of H-9 revealed only the enhancements of signals H-11 and H-12 on ring B. Since the amplification of the signal only provided enhancement of the proton signals on the same aromatic ring, it can be suggested that triclosan is locked into a stereospecific conformation.

HMBC was then utilized for completion of the assignments and correlations. It must be noted that in order to do this, all peaks assigned so far must be used- Assignment of H-1, H-4, H-5, H-9, H-11 and H-12 and all corresponding carbon signals.

The HMBC (Figure 21) showed strong J^3 but weak J^2 signals. The assignment of C2 was evaluated through the strong H4-C2 J^3 correlation and the weak H1-C2 J^2 correlation. The chemical shift for C2, 147.42ppm, agreed with the calculated[44] ^{13}C chemical shift of 147.1 ppm. The next signal analyzed for assignment was at 150. 49 ppm, C7. It showed weak H12-C7 J^2 correlation, strong H11-C7 J^3 correlation, and strong H9-C7 J^3 correlation. The calculated[44] ^{13}C chemical shift for C7 was 155.7 ppm which is congruent to the actual chemical shift. Working downfield, the assignment of C3 was made by the weak H4-C3 J^2 correlation, strong H5-C3 J^3 correlation, and strong H1-C3 J^3 correlation. The chemical shift for C3 was 142.25 ppm, which is in agreement with the calculated[44] ^{13}C chemical shift of 142.8 ppm. Continuing downfield, the next signal analyzed was C10. It showed weak H11-

C10 J^2 correlation, weak H9-C10 J^2 correlation, and strong H12-C10 J^3 correlation. The chemical shift for C10 was 130.24 ppm, which is in agreement with the calculated[44] ^{13}C chemical shift of 130.6 ppm. Assignment was then made for C6. The weak H1-C6 J^2 correlation, weak H5-C6 J^2 correlation, and strong H4-C6 J^3 correlation were evaluated along with the chemical shift for C6 of 129.96 ppm, which is in agreement with the calculated[44] ^{13}C chemical shift of 131.2 ppm. Lastly, the assignment of C8 was made through the observation of the strong H12-C8 J^3 correlation and the weak H9-C8 J^2 correlation. Again, the chemical shift for C8 of 126.39 ppm, which is in agreement with the calculated[44] ^{13}C chemical shift of 120.6 ppm. Cadmium nitrate was then added to the sample to reduce the amount of hydrogen bonding between the hydroxyl group hydrogen. The HMBC was rerun to determine the correlating assignment of the signal (Figure 22). The spectrum demonstrated assignment of OH(2) by correlations of J^2 OH(2)-C2 and J^3 OH(2)-C3, OH(2)-C2.

The comparison of the two methods' assignments, actual by the JEOL ECX 400 MHz NMR and theoretical by Spartan '04 (Table 7), demonstrated the consistency in lower chemical shifts on behalf of Spartan '04 when compared to the actual chemical shifts values obtained by the JEOL ECX 400 MHz NMR. While Spartan '04 serves as a useful tool for projecting an NMR spectra, if an instrument is available for use, it should be the preferred method as seen by the comparison.

CONCLUSION

The presence of triclosan was detected in influent wastewaters of Central Virginia at notable concentrations. Detection of the triclosan shows the use and then subsequent release

of triclosan into the environment. Some of these concentrations are significantly greater than the toxicity levels of specific aquatic organisms. Such levels could ultimately impact the food web and conditions of the aquatic systems due to the loss of these organisms. Several triclosan-resistant strains were isolated and identified from the wastewater samples. The potential does exist that increased exposure to antimicrobials such as triclosan could lead to antibiotic resistance. A complete proton and carbon assignment by nuclear magnetic resonance (NMR) was also performed due to the inability to find such assignments after performing a complete literature search. While the overall fate of triclosan remains unknown, preliminary assessments of the concentrations of triclosan and bacteria demonstrating resistance to triclosan in the environment should be established should this issue later develop into a public health concern.

WORKS CITED

1. Koplin, D.W.; Fulong, E.T.; Meyer, M.T.; Thurman, E.M.; Zaugg, S.D.; Barber, L.B.; Buxton, H.T. *Environ. Sci. Technol.* **2002**, *36*, 1202-1211.
2. Anderson, P.D.; D'Aco, V.J.; Shanahan, P.; Chapra, S.C.; Buzby, M.E.; Cunningham, V.L.; Duplessie, B.M.; Hayes, E.P.; Mastrocco, F.J.; Parke, N.J.; Rader, J.C.; Samuelian, J.H.; Schwab, B.W. *Environ. Sci. Technol.* **2004**, *38*, 838-849.
3. Buser, H.R.; Poiger, T.; Muller, M.D. *Environ. Sci. Technol.* **1998**, *32*, 3449-3456.
4. Buser, H.R.; Poiger, T.; Muller, M.D. *Environ. Sci. Technol.* **1999,** *33,* 2529-2535.
5. Wilson, B.A.; Smith, V.H.; Denoyelles, F. Larive, C.K. *Environ. Sci. Technol.* **2003**, *37*, 1713-1719.
6. U.S. Environmental Protection Agency. *Impacts and Control of SCOs and SSOs*; Report No. EPA 833-R-04-001; U.S.EPA, Office of Water: Washington, DC, 2004.
7. Singer, H.; Muller, S.; Tixier, C.; Pillonel, L. *Environ. Sci. Technol.* **2002**, *36*, 4998-5004.
8. Bhargava, J.G.; Howes, D.; Rutherford, T. *Am. J. Infect. Control.* **1996**, *24*, 209-218.
9. Cox, A.R. *J. Soc. Cosmet. Chem.* **1987**, *38*, 223-231.
10. DeSalva, S.J.D.; Kong, B.M.; Lin., Y.J. *Am. J. Dent.* **1989**, *2*, 185-196.
11. Daughton, C.G.; Ternes, T. *Environ. Health Perspet.* **1999**, *107*, 907-937.
12. Reiss, R.; Nackay, N.; Habid, C.; Griffin, J. *Environ. Toxicol. Chem.* **2002**, *21*, 2483-2492.
13. Glaser, A. *Pesticides and You.* **2004**, *24*, 9-17.

14. Reither, L.L. "*Detection of Triclosan in Geographically Diverse Influent Wastewater Samples and the Isolation of Triclosan-Resistant Bacteria.*" Sweet Briar College Senior Honors Thesis. 2002.

15. Kummerer, K. *J. Antimicrobial Chemo.* **2004**, *54*, 311-320.

16. Heath,R.J.; Yu, Y.T.; Shapiro,M.A.; Olson,E.; Rock,C.O. *J. of Biological Chemistry.* **1998**, *273*,30316-30320.

17. McMurry, L.M.; Oethinger, M.; Levy, S.B. *Nature.* **1998**, *34*, 531-532.

18. Parikh, S.L.; Xiao, G.; Tonge, P.J. *Biochemistry.* **2000**, *39*, 7645-7650.

19. Heath,R.J.; Rubin,J.R.; Holland,D.R.; Zhang,E.; Snow,M.E.; Rock,C.O. *J. of Biological Chemistry.* **1999**,*274*,11110-11114.

20. Levy,C.W.; Roujeinikova, A.; Sedelnikova, S.; Baker,P.J.; Stuitje,A.R.; Slabas,A.R.; Rice,D.W.; Rafferty,J.B. *Nature.* **1999**,*398*,383-384.

21. Kapoor,M.; Mukhi,P.L.S.; Surolia,N.; Suguna,K.; Surolia,A. *J. Biochem.* **2004**,*381*,725-733.

22. Orvos, D.R.; Versteeg, D.J.; Inauen, J.; Capdevielle, M.; Rothenstein, A.; Cunningham,V. *Environ. Toxicol. Chem.* **2002**, *21*, 1338-1349.

23. Lindstrom, A.; Buerge, I.J.; Poiger, T.; Bergqvist, P.E.; Muller, M.D.; Buser, H.R. *Environ. Sci. Technol.* **2002**, *36*, 2322-2329.

24. Balmer, M.E.; Poiger, T.; Droz, C.; Fomanin, K.; Bergqvist, P.E.; Muller, M.D.; Buser, H.R. *Environ. Sci. Technol.* **2004**, *38*, 390-395.

25. Fraker, S.L.; Smith, G.R. *Environ. Toxicol. Chem.* **2004**, *19*, 250-256.

26. Zhang, H.; Huang, C.H. *Environ. Sci. Technol.* **2003,** *37*, 2421-2430.

27. Sworfford, W.; Vikesland, P. *Letters. Environ. Sci. Technol.* **2005**, 271A-272A.

28. Rule, K.L.; Evvett, V.R.; Vikesland, P.J. *Environ. Sci. Technol.* **2005**, *39*, 3176-3185.

29. Miyazaki,T.; Yamagishi,T.; Matsumota,M. *Bull. Environ. Contam. Toxicol.* **1984**, *32*, 227-232.

30. Guillen,J.; Bernabeu, A.; Shapiro,S.; Villalain,J. *European J. Biophysics*. **2004**, *33*, 448-453.

31. Latch,D.E.; Packer,J.L.; Arnold,W.A.; McNeill,K. *J. of Photochem. and Photobio.* **2003**, *158*, 63-66.

32. Duan,M.S.; Zhao,N.; Ossurardottir,I.B.; Thorsteinsson,T.; Loftsson,T. *International J. of Pharmaceutics*. **2005**, *297,* 213-222.

33. Maestrelli,F.; Mura,P.; Alonso,M.J. *J. of Microencapsulation*. **2004**,*21*,857-864.

34. Greenberg, A.E., L.S. Clesceri, and A.D.Eaton. (eds.) Standard Methods for the Examination of Water and Wastewater. 18th. EPS Group: Baltimore. **1992**.

35. Prescott,L.M.; Harley,J.P.; Klein,D.A. Microbiology, 6th Ed. McGraw-Hill: New York. **2005**.

36. Sankaran,N. Microbes and People. Oryx Press: Phoenix. **2000**.

37. Hovander, L.; malmberg,T.; Athanasiadou,M.; Athanassiadis,I.; Rahm,S.; Bergman, A.; Klasson Wehler,E. *Environ. Contamin. & Tox*. **2002**, *42*,105-117.

38. Adolfsson-Erici, M.; Pettersson, M.; Parkkonen, J. ; Sturve, J. *Organohalogen Compounds*. **2000**, *45*, 83-86.

39. Gary, J.D.; Sato,T.K.; Stefan,C.J.; Bonangelino,C.J.; Weisman,L.S.; Emr,S.D. *Mol. Biol. Cell.* **2002**, *13*, 1238-1251.

40. DeWald, D. *Curr. Biol*. **2002**, *12*, R491.

41. Dove,S.K.; McEwen,R.K.; Mayes,A.; Hughes,D.C.; Beggs,J.D.; Nichell,R.H. *Curr. Biol.* **2002**, *12*, 885-893.

42. Bonangelino, C.J.; Nau, J.J.; Duex, J.E.; Brinkman, M.; Wurmser, A.E.; Gary, J.D.; Emr, S.D.; Weisman, L.S. *J. Cell. Biol.* **2002**, *156*, 1015-1028.

43. Fraise, A.P. *J. Antimicrobial Chemotherapy*. **2002**, *49*, 11-12.

44. Silverstein, R.M.; Webster, F.X.; Kiemle, D.J. Spectrometric Identification of Organic Compounds, 7th Ed. John Wiley & Sons, Inc: New Jersey. **2005**.

Table 2. Triclosan concentrations calculated from HPLC collected data of extracted primary influent samples.

Wastewater Treatment Plant	Mean Triclosan retention time (min)	Mean triclosan concentration (ug/L)	Standard deviation of mean concentration (ug/L)
Rivanna Wastewater Treatment Plant at Moores Creek, Charlottesville, VA (overall)	10.05	35.0	48.9
Sampling of 27 February 2006		110.0	9.2
Sampling of 20 January 2006		3.7	0.55
Amherst County Wastewater Treatment Plant, Amherst, VA (overall)	9.92	0.99	0.22
Sampling of 27 February 2006		0.98	0.35
Sampling of 20 January 2006		0.95	0.25

Table 3. Individual isolated strain results for gram stain, oxidase test, TSI slant, absorbance for BIOLOG density and morphology.

	GP/GN	morph	type	tsi	oxi +/-	abs	BIOLOG
AMHERST							
A-C-1	GN	C/R	GNNENT	NC/NC	P	0.265	
A-C-2	GN	R	GNENT	NC/NC,GOS	N	0.12	FP
A-10-1	GN	R	GNENT	NC/NC	N	0.129	
A-15-1	GN	R	GNENT	K/NC	N	0.126	FP
CVILLE							
C-C-1	GP	R	GPR/C	NC/NC,GOS	N	0.948	
C-C-2	GP	R	GPR/C	NC/NC,GOS	N	0.923	FP
C-C-3	GP	R	GPR/C	NC/NC,GOS	P	0.917	FP
C-10-1	GN	C	GNNENT	K/NC	P	0.27	
C-10-2	GN	R	GNENT	NC/NC	N	0.119	
C-10-3	GN	R	GNENT	NC/NC	N	0.12	
C-15-1	GN	R	GNNENT	K/K	P	0.268	
C-15-2	GN	R	GNENT	NC/NC	N	0.125	TLI
C-15-3	GN	C	GNENT	K/NC	N	0.128	FP
C-15-4	GN	R	GNENT	K/NC	N	0.118	FP
C-20-1	GN	R	GNENT	NC/NC	N	0.129	
C-20-2	GN	R	GNENT	NC/NC	N	0.131	FP
C-20-3	GP	R	GPR/C	NC/NC	N	0.922	
C-20-4	GN	R	GNENT	NC/NC,GOS	N	0.121	FP
C-20-5	GN	R	GNENT	K/NC	N	0.117	TLI
C-50-1	GN	R	GNNENT	K/NC	P	0.262	
C-50-2	GN	R	GNENT	NC/NC	N	0.127	TLI
C-50-3	GP	R	GPR/C	K/NC,GOS	N	0.944	FP
C-50-4	GP	R	GPR/C	NC/NC,GOS	N	0.937	FP

Morph-colony morphology: C-cocci, R-rod
Type-BIOLOG type:
TSI-Triple Sugar Iron Result: NC/NC-no change in slant/butt, K/NC-catabolized aerobically with alkaline product, K/K-catabolized aerobically and anaerobically with alkaline product, GOS-Growth On Surface of slant
Oxi+/-: Oxidase test result: P-positive result, N-negative
Abs-Measured absorbance: Inoculum density recommended for BIOLOG plating

Table 4. Resistant strains identified by BIOLOG.

Wastewater Treatment Plant	Bacteria Identified by BIOLOG
Rivanna Wastewater Treatment Plant at Moores Creek, Charlottesville, VA	*Serratia rubidaea*
Rivanna Wastewater Treatment Plant at Moores Creek, Charlottesville, VA	*Corynebacterium nitrilophilus*
Rivanna Wastewater Treatment Plant at Moores Creek, Charlottesville, VA	*Brevibacterium mcbrellneri*
Rivanna Wastewater Treatment Plant at Moores Creek, Charlottesville, VA	*Brevundimonas vesicularis*
Rivanna Wastewater Treatment Plant at Moores Creek, Charlottesville, VA	*Raoultella planticola*
Amherst County Wastewater Treatment Plant, Amherst, VA	*Burkholderia glumae*

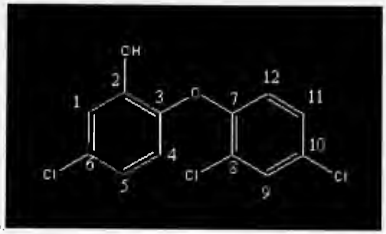
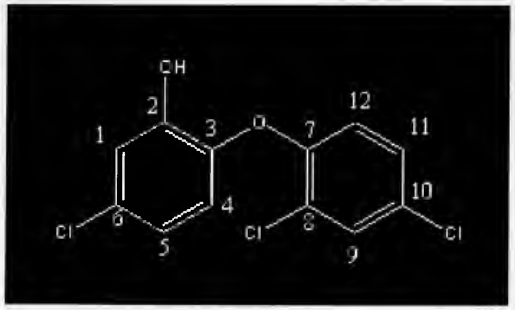


Table 5. ^{1}H & ^{13}C NMR assignments for triclosan in $CDCl_3$

Position	^{1}H δ	Multiplicity	J (Hz)	Integration	^{13}C δ	^{13}C δ (Calculated)[44]
1	7.06	d	2.29	1 H	117.01	117.6
2	-	-	-	-	147.42	147.1
3	-	-	-	-	142.25	142.8
4	6.65	d	8.24	1 H	118.13	122.1
5	6.81	dd	2.29,8.24	1 H	120.73	123.0
6	-	-	-	-	129.96	131.2
7	-	-	-	-	150.49	155.7
8	-	-	-	-	126.39	120.6
9	7.47	d	2.29	1 H	130.83	130.5
10	-	-	-	-	130.24	130.6
11	7.22	dd	2.75,8.7	1 H	128.36	128.3
12	6.94	d	9.16	1 H	120.92	121.1
OH (2)	5.6	bs	-	1 H		

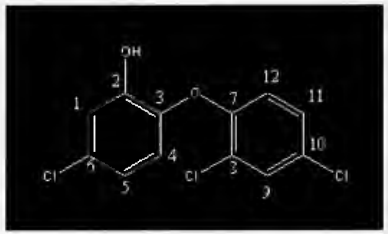
^{1}H NMR, 400 MHz and referenced to residual $CHCl_3$ 7.23 ppm; ^{13}C NMR, 100 MHz and referenced to $CDCl_3$ 77.33 ppm

Table 6. 1H and 13C NMR assignments for triclosan by Spartan '04.

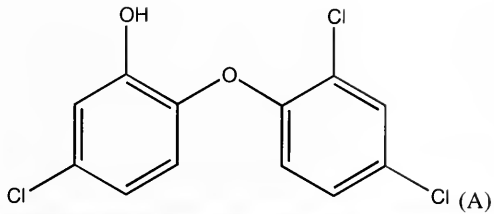


Position	^{1}H δ	^{13}C δ
1	7.127	111.789
2	-	138.951
3	-	131.371
4	6.939	106.575
5	6.858	111.677
6	-	122.783
7	-	139.782
8	-	115.838
9	7.447	125.583
10	-	117.823
11	7.367	122.742
12	7.000	105.535
OH (2)	5.977	

Table 7. Comparison of NMR assignments for triclosan by JEOL ECX 400 MHz and Spartan '04.



Position	Spartan ^{1}H δ	JEOL ^{1}H δ	Spartan ^{13}C δ	JEOL ^{13}C δ
1	7.127	7.06	111.789	117.01
2	-	-	138.951	147.42
3	-	-	131.371	142.25
4	6.939	6.65	106.575	118.13
5	6.858	6.81	111.677	120.73
6	-	-	122.783	129.96
7	-	-	139.782	150.49
8	-	-	115.838	126.39
9	7.447	7.47	125.583	130.83
10	-	-	117.823	130.24
11	7.367	7.22	122.742	128.36
12	7.000	6.94	105.535	120.92
OH (2)	5.977	5.6		



(A)

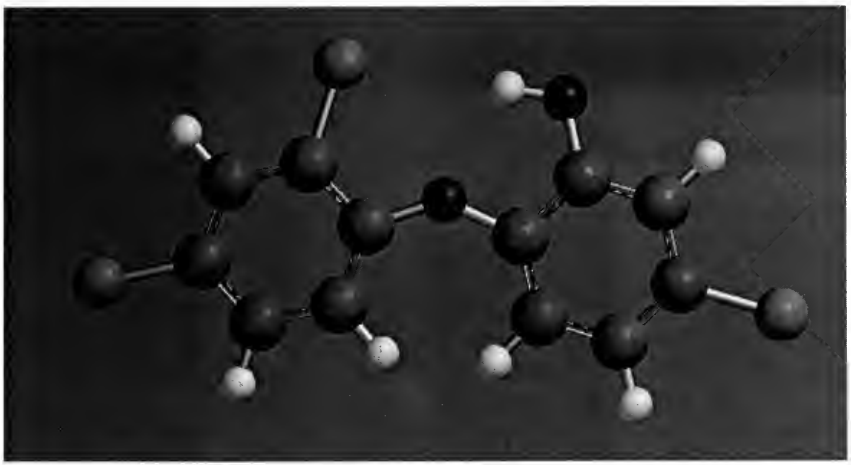
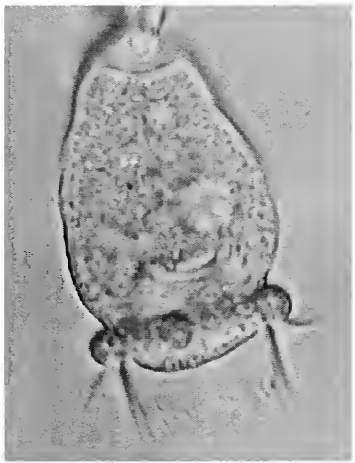
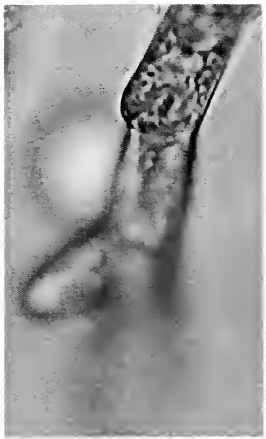

(B)

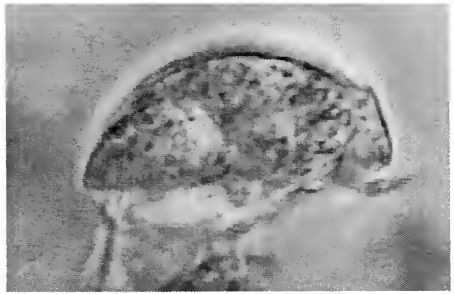
Figure 1. ChemDraw Structure of Triclosan (A) and Spartan '04 model structure of Triclosan (B).



Rotifera epiphanes



Chlorophyta microthamnion



Daphnia magna

Figure 5. Microflora identified in wastewater samples.

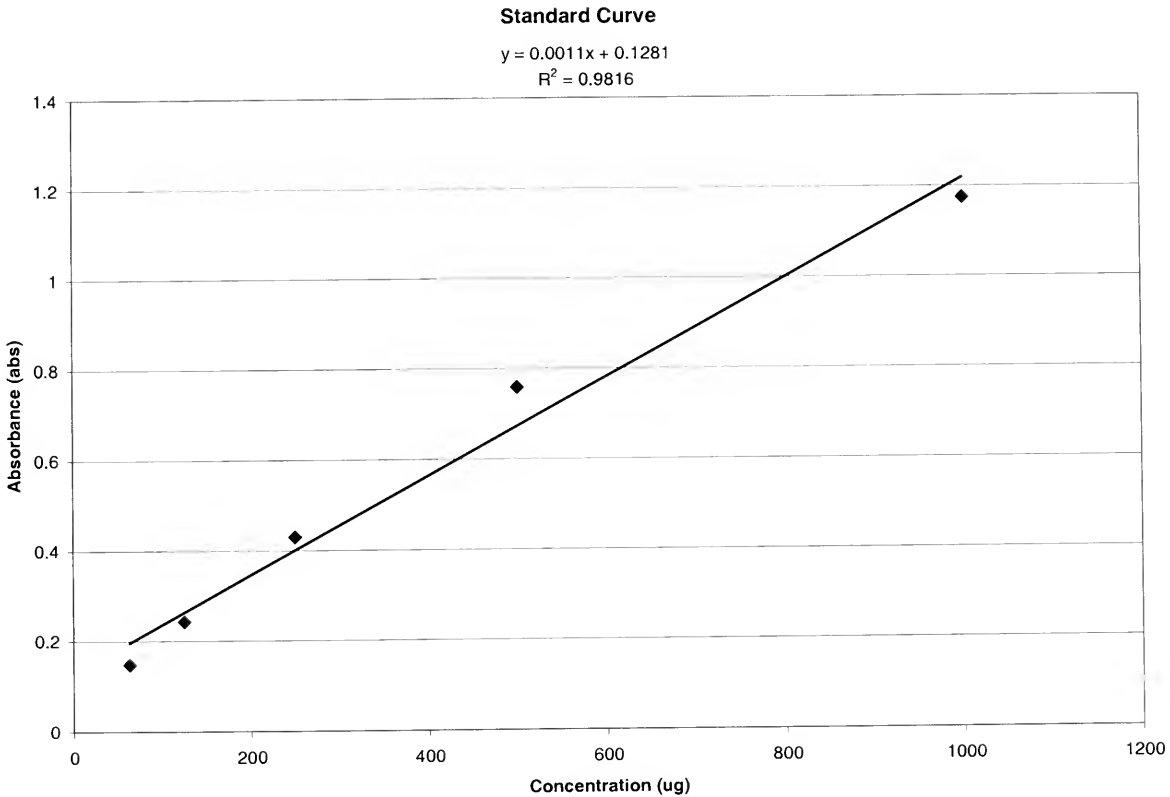


Figure 6. Standard curve of zinc, copper and nickel by atomic absorption spectrometry.

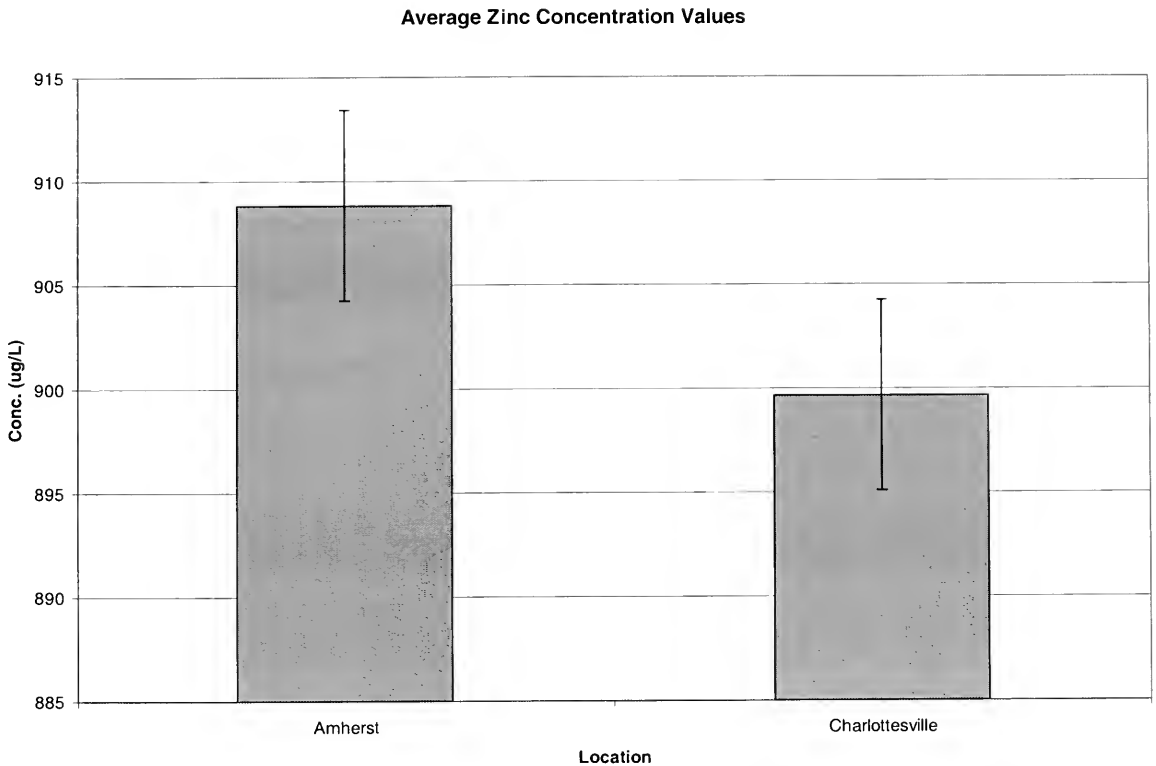


Figure 7. Average concentration of zinc found in wastewater samples. Error bars represent standard error.

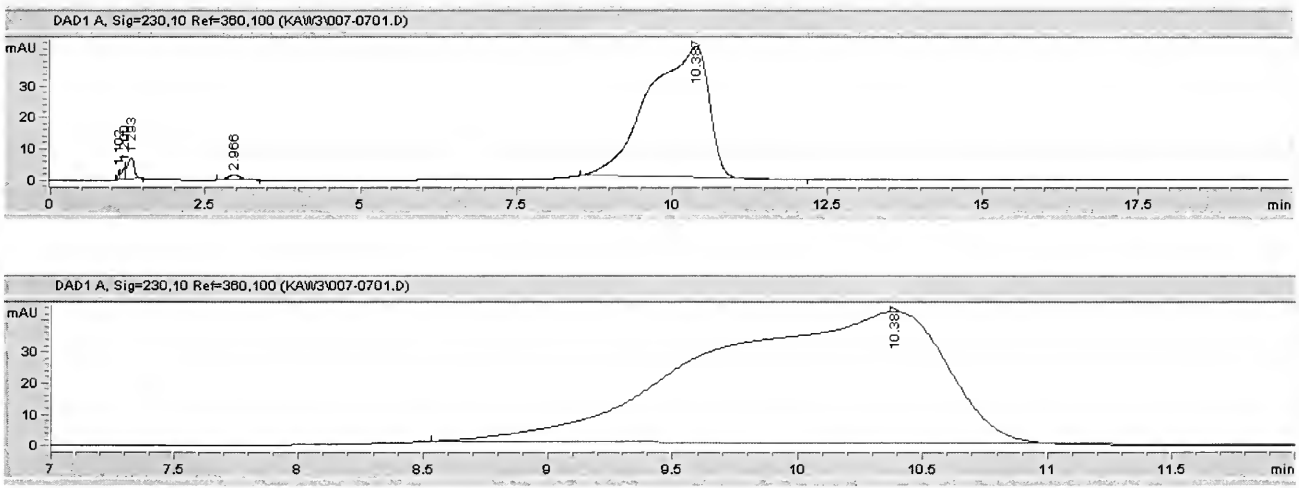


Figure 8. HPLC Chromatogram of the triclosan standard (top) and magnified image of peak (bottom). The retention time is 10.4 minutes.

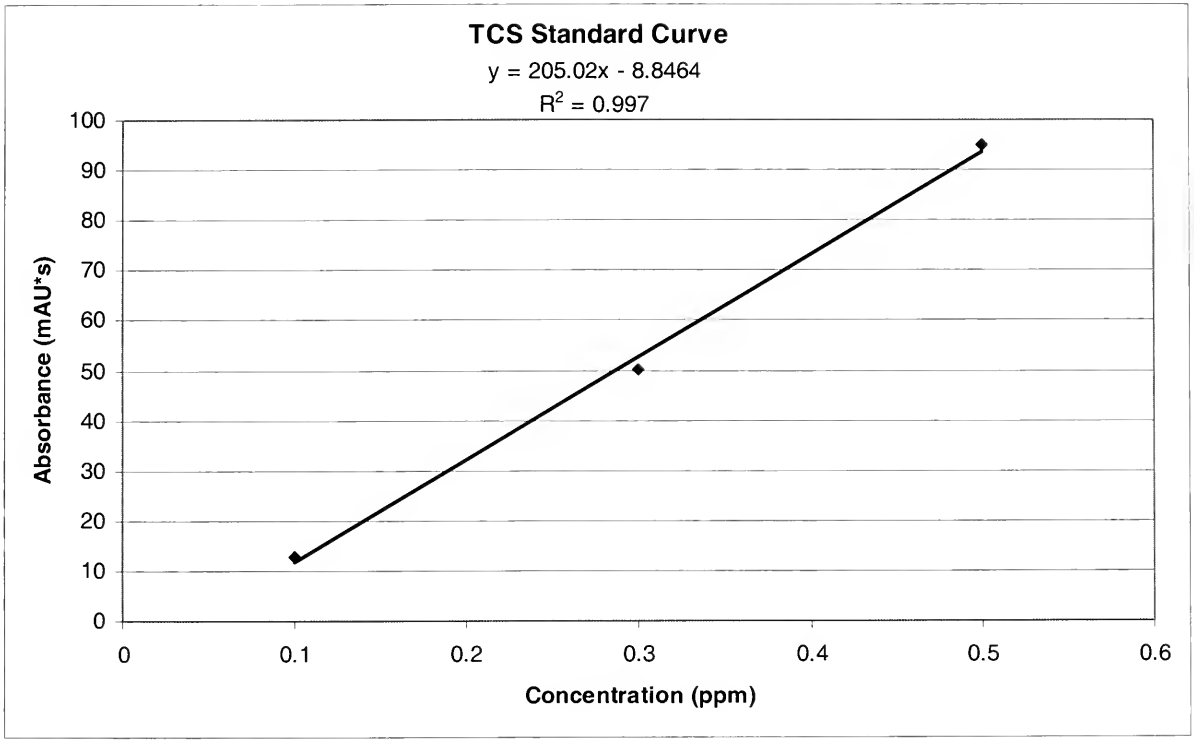


Figure 9. Standard curve of triclosan.

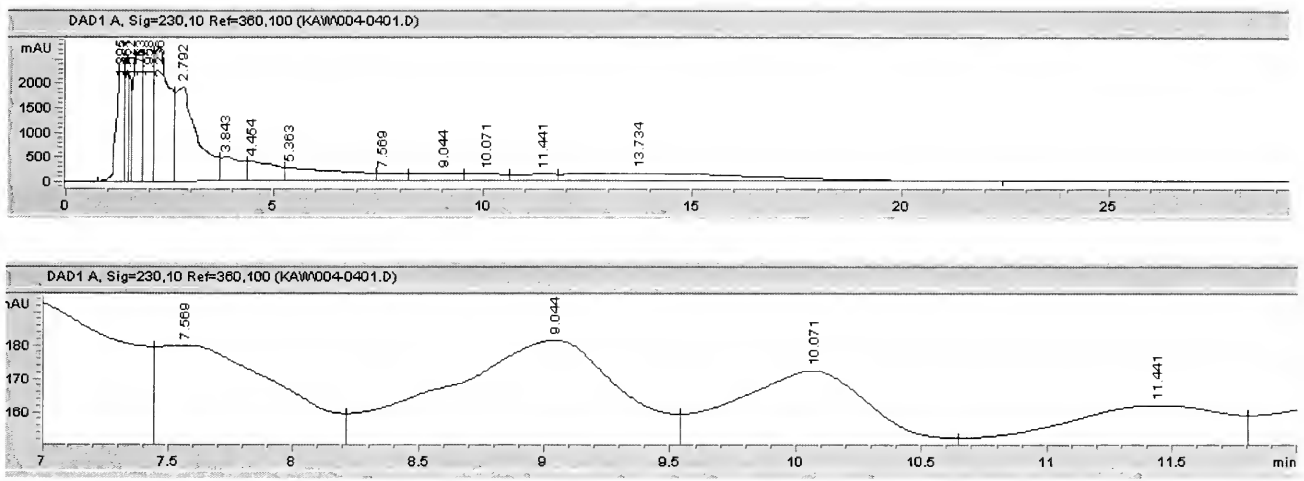


Figure 10. Representative HPLC chromatogram of the extracted primary influent from the Rivanna Wastewater Treatment Plant at Moores Creek in Charlottesville, VA (top) and magnified image highlighting the triclosan peak (bottom). The retention time of the triclosan is 10.07 minutes.

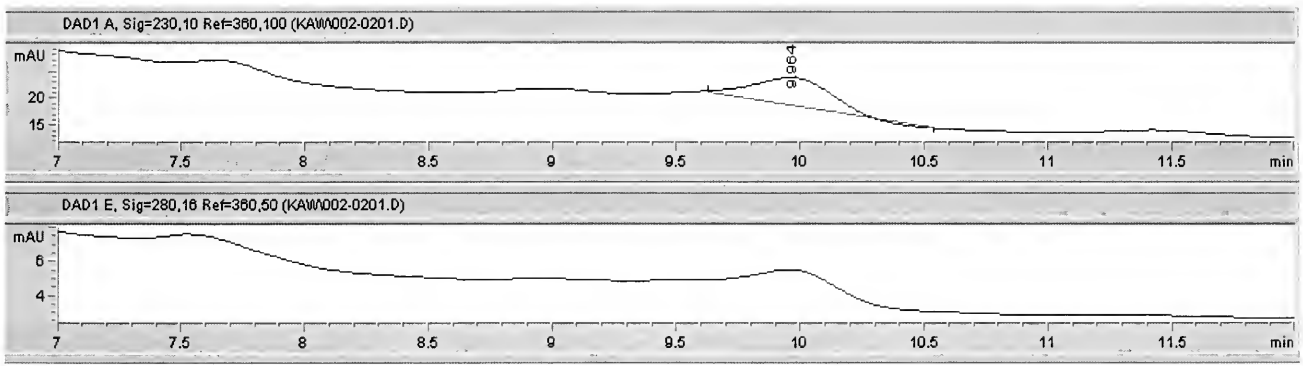


Figure 11. Representative HPLC chromatogram of the extracted primary influent from the Amherst County Wastewater Treatment Plant in Amherst, VA. The retention time of triclosan is 9.96 minutes.

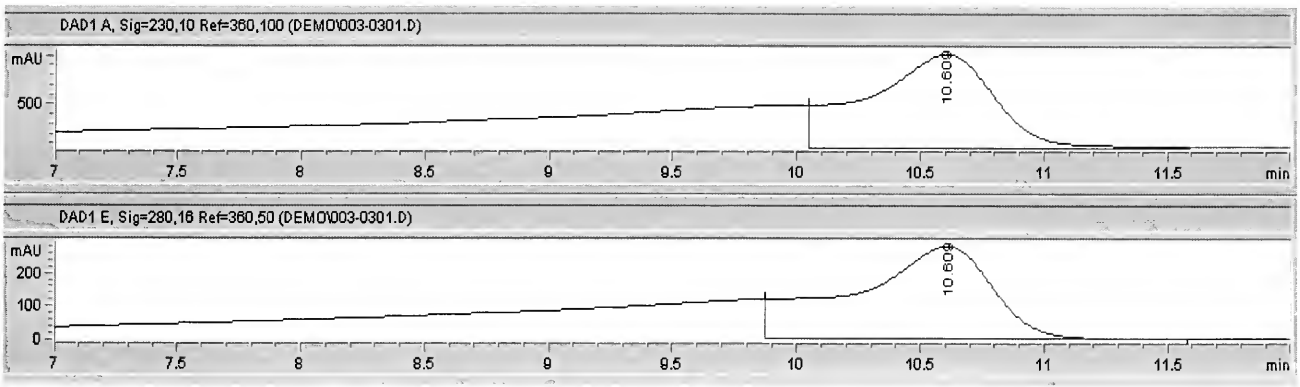


Figure 12. Representative HPLC chromatogram of the extracted primary influent from Rivanna Wastewater Treatment Plant at Moores Creek in Charlottesville, VA with triclosan spike. The retention time of the triclosan is 10.6 minutes.

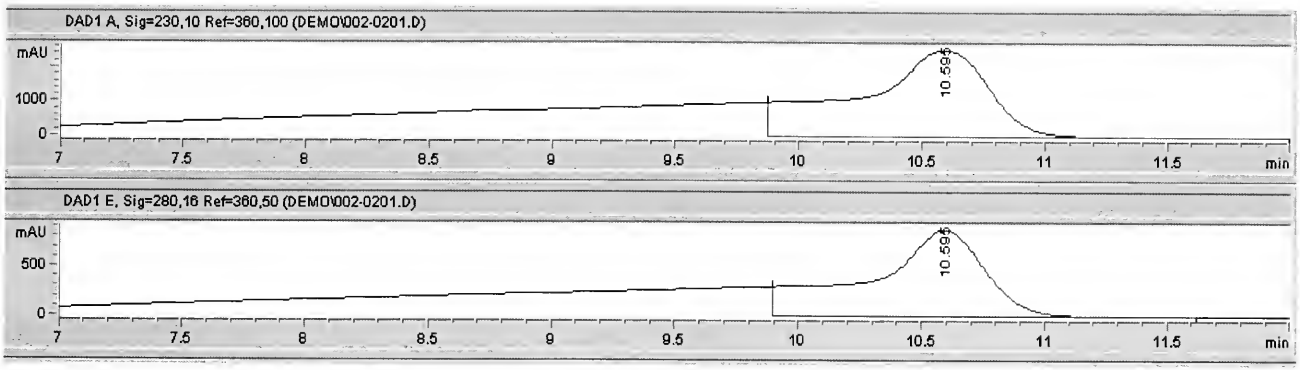


Figure 13. Representative HPLC chromatogram of the extracted primary influent from Amherst Wastewater Treatment Plant Amherst, VA with triclosan spike. The retention time of the triclosan is 10.6 minutes.

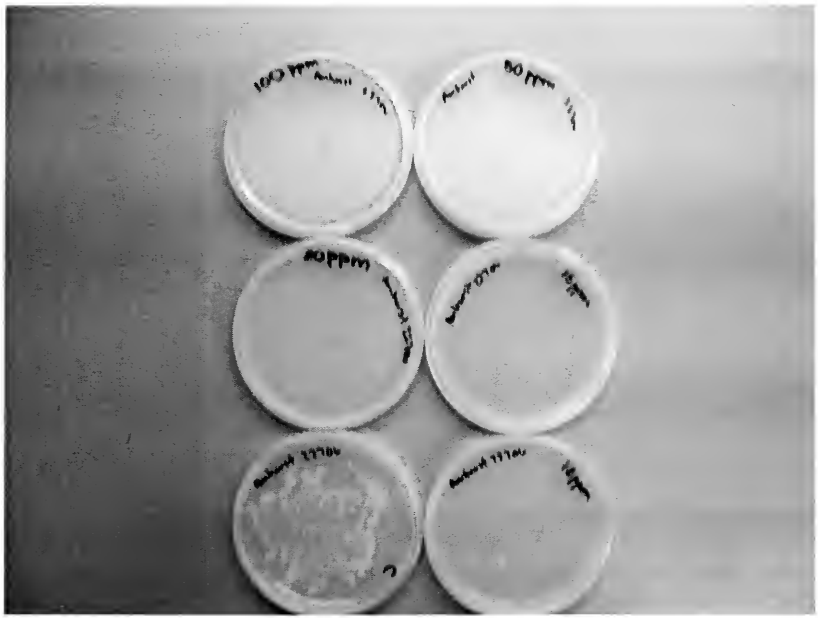


Figure 14. R2A agar plates enriched with triclosan showing visible colony growth and therefore bacterial resistance to triclosan from the Amherst wastewater sample.

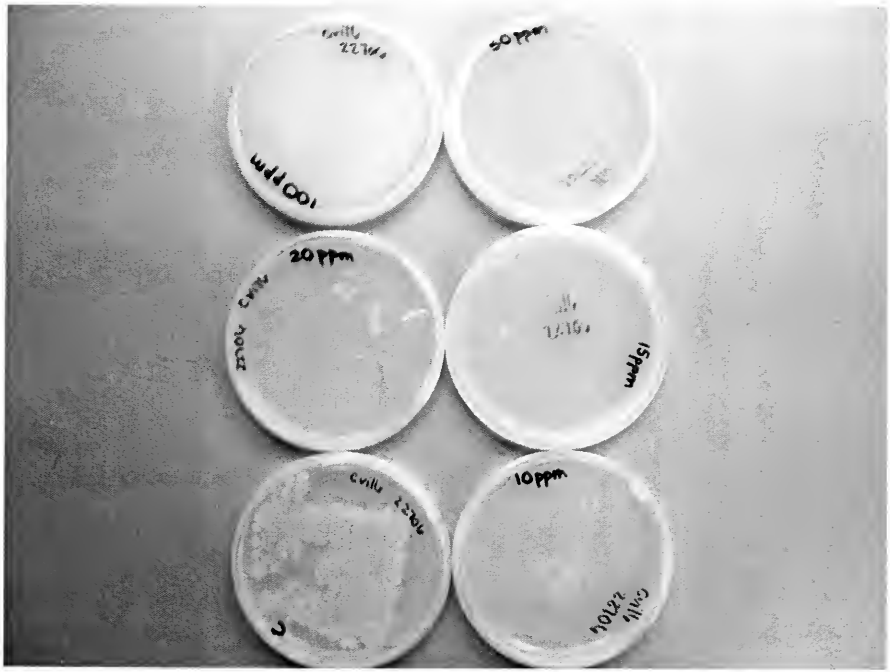


Figure 15. R2A agar plates enriched with triclosan showing visible colony growth and therefore bacterial resistance to triclosan from the Rivanna Moores Creek in Charlottesville wastewater sample.

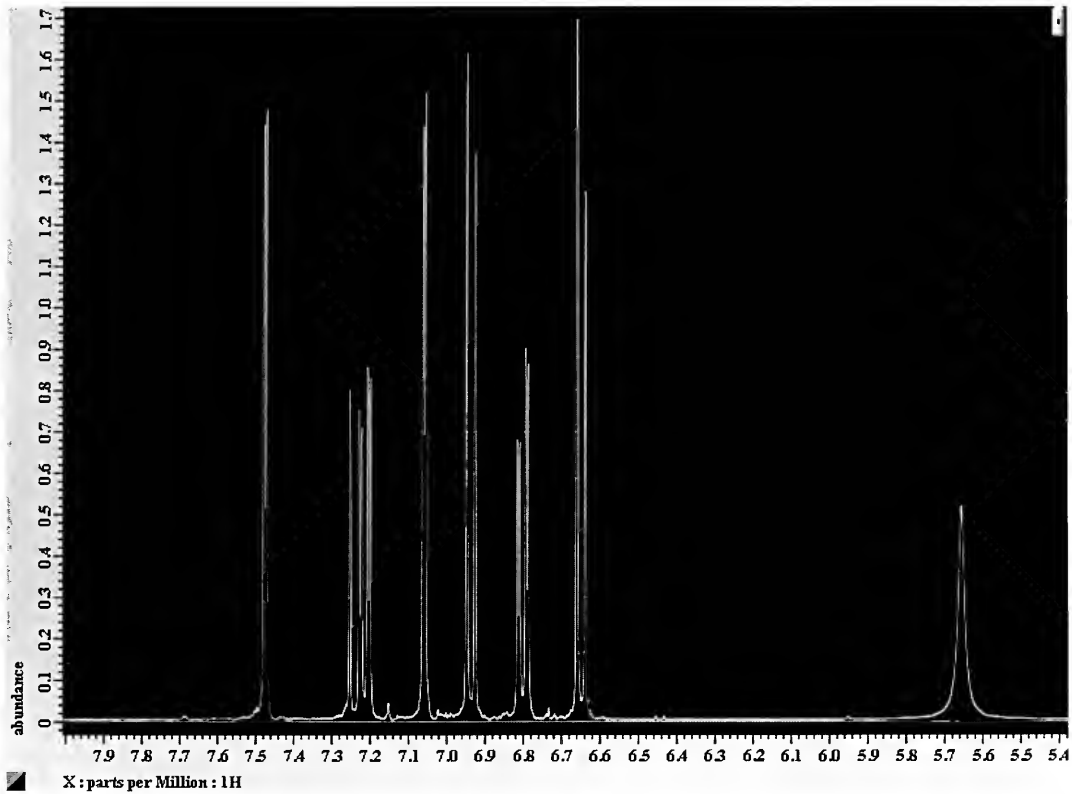


Figure 16. ^{1}H NMR spectrum of triclosan.

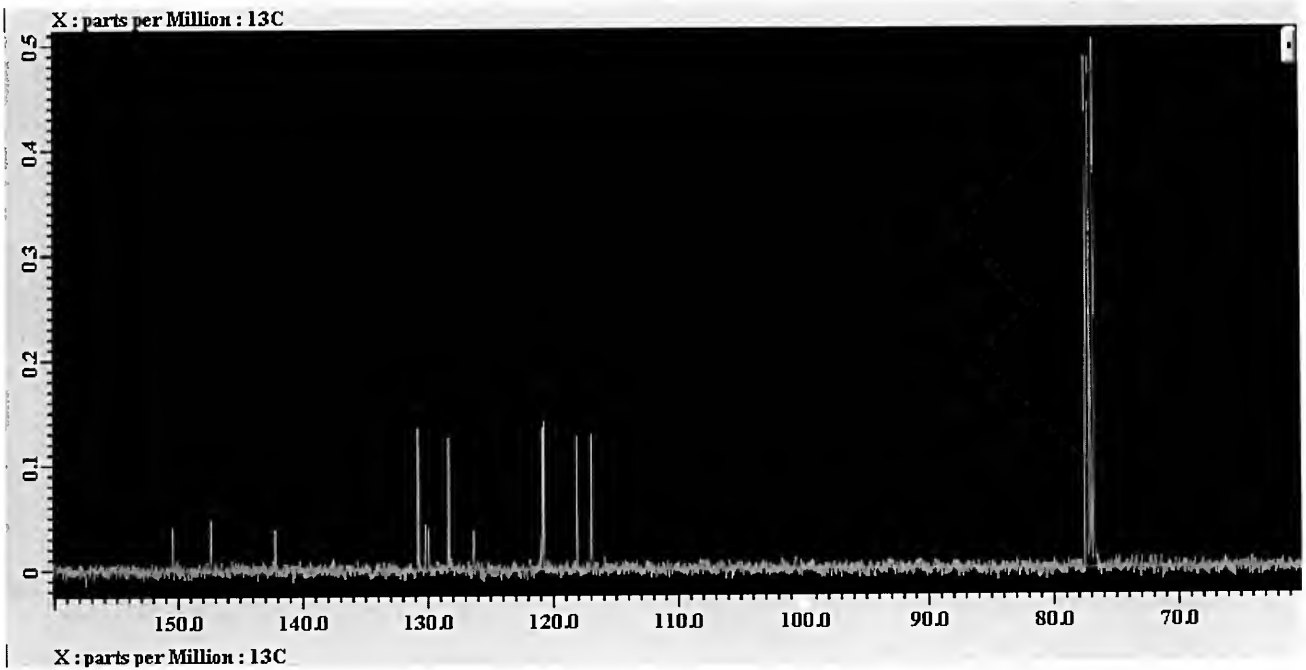


Figure 17. ^{13}C NMR spectrum of triclosan.

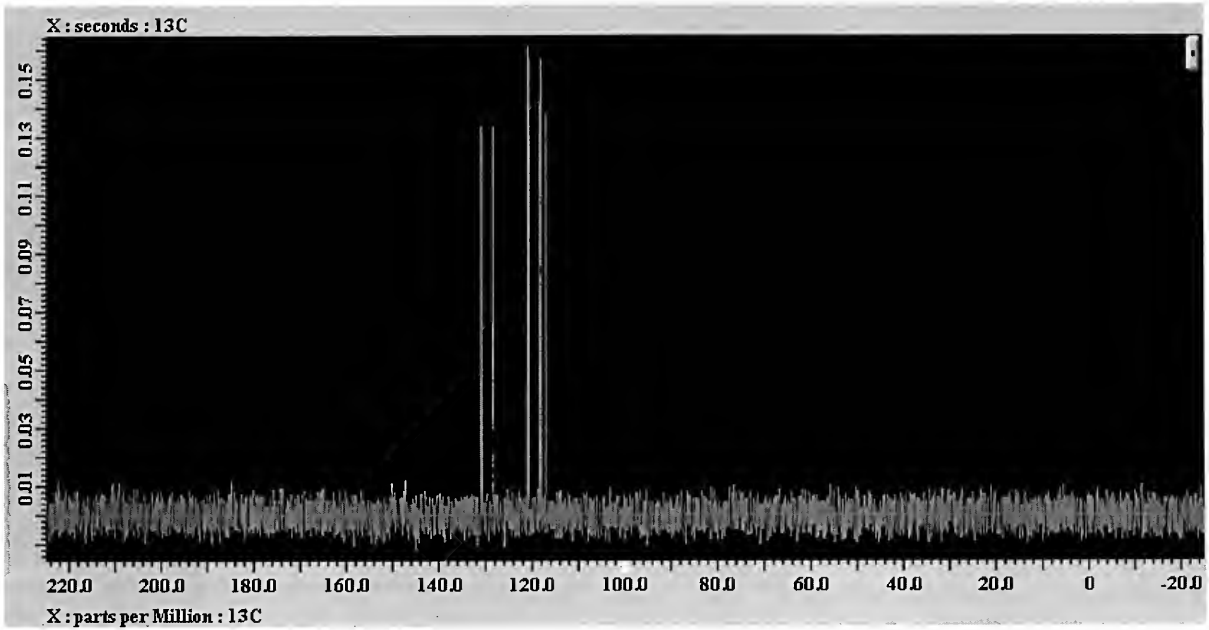


Figure 18. DEPT 135 NMR of triclosan.

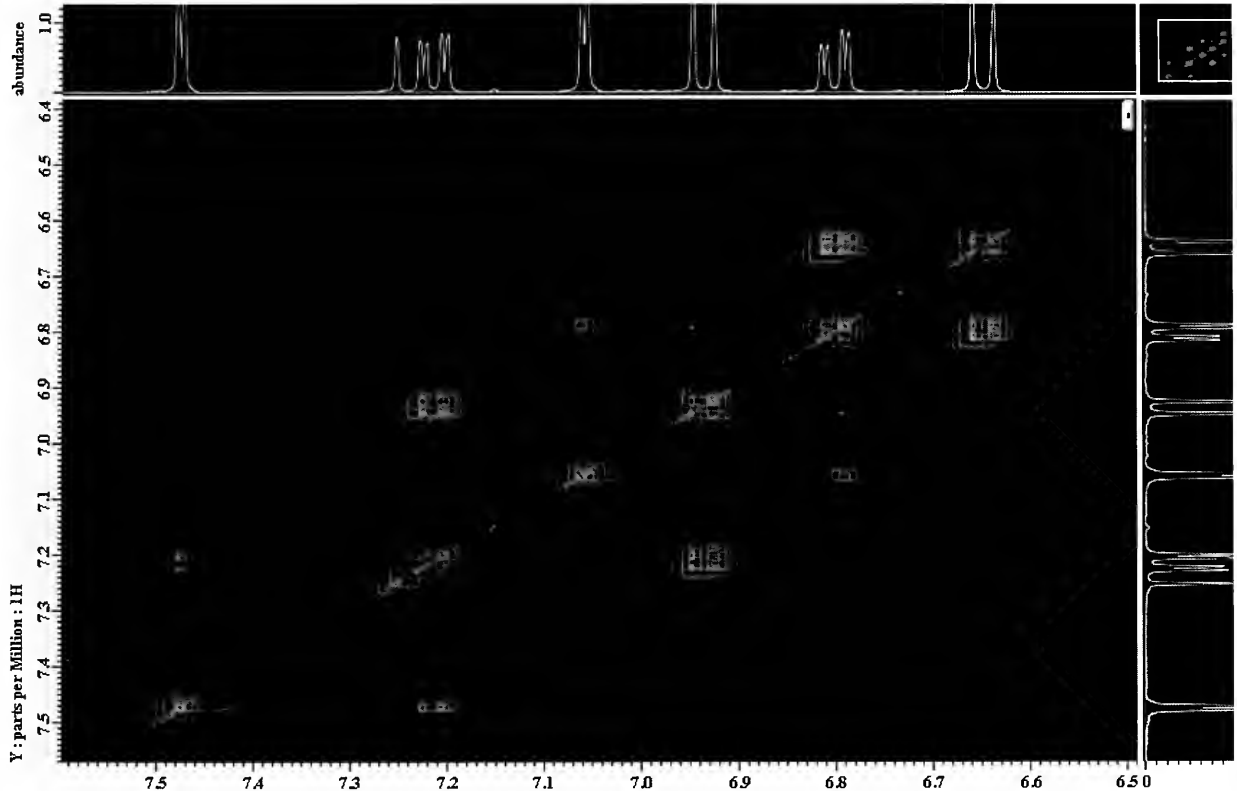


Figure 19. ^{1}H-^{1}H COSY NMR of triclosan.

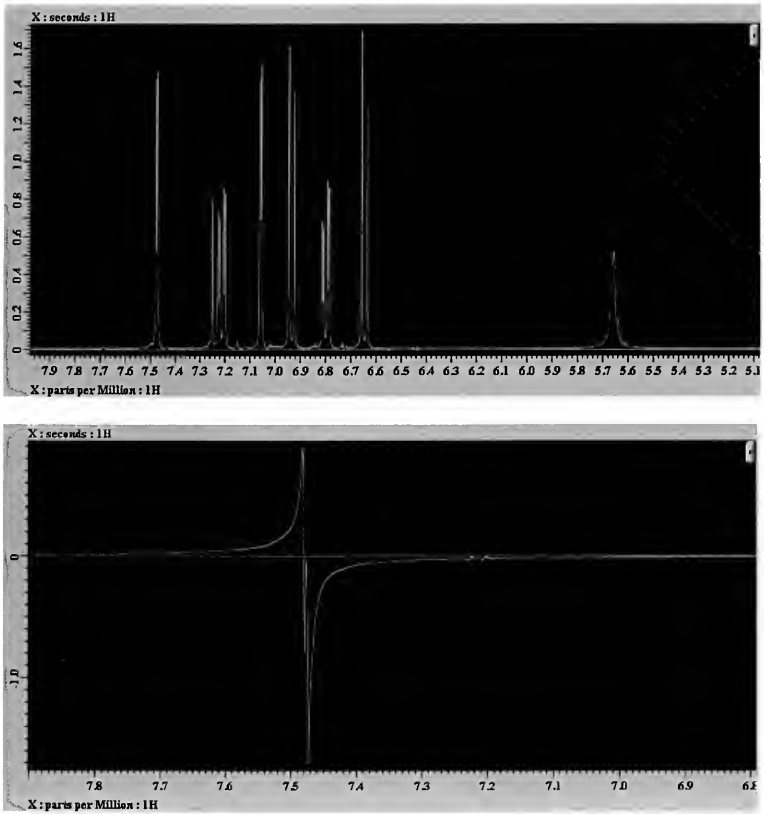


Figure 20. ^{1}H NMR (top) and nOe irradiation of H-9 (bottom) of triclosan.

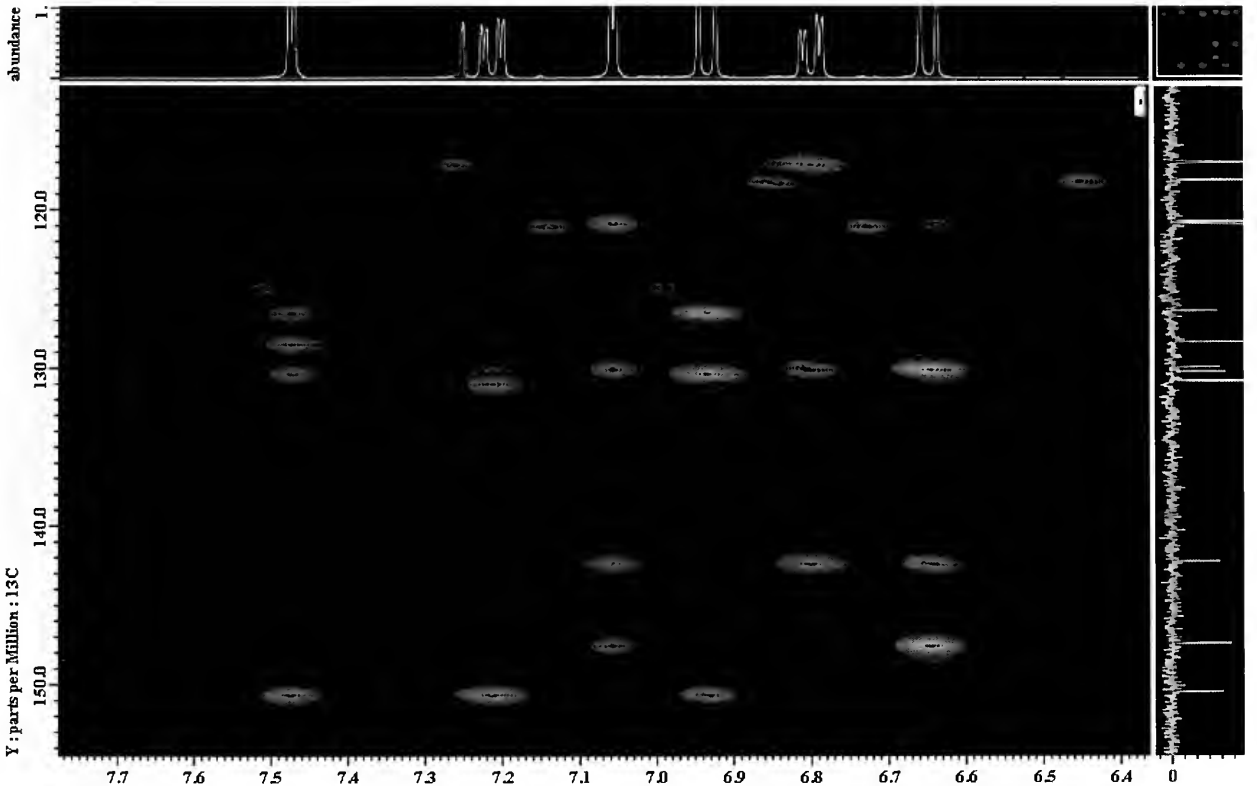


Figure 21. HMBC NMR of triclosan.

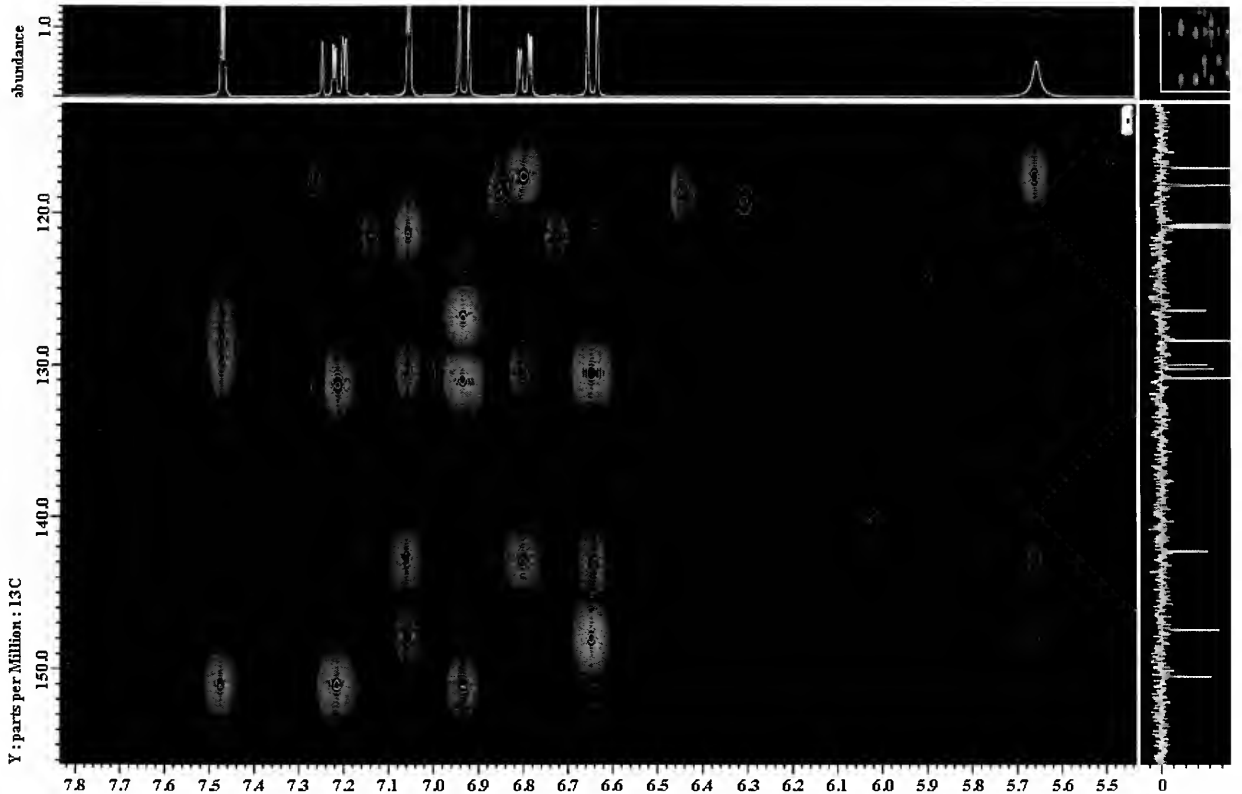


Figure 22. HMBC NMR spectrum, with $Cd(NO_3)_2$ added, of triclosan.